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The purpose of this case-control study is to investigate the association between genetic and encocrine markers of estrogen metabolism and prostate cancer progression. Androgens (e.g., testosterone) may be critical in prostate cancercinogenesis, but there is accumulating evidence that estrogens facilitate progress during the later stages of prostate cancer formation [1-4]. To explore the role of estrogens in human prostate carcinogenesis, we proposed to investigate the association between genetic and endocrine markers of estrogen metabolism and the detection of high-grade prostatic intraepithelial neoplasia (HGPIN) and stage I/II/III prostate cancer. The first project year included protocol development and IRB approval, and the second year focused on subject recruitment and data collection. The third year focused on recruitment, data collection, and analysis. Specific accomplishments include recruitment of 489 subjects to the protocol (90% of eligibles). We have conducted several analyses looking at the association between genetic variants and prostate cancer. We have exceeded recruitment goals, and at this time have met almost all data collection and processing goals. Further details provided below are in parallel with the statement of work.

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MOLECULAR MARKERS OF ESTROGEN METABOLISM AND PROGRESSION FROM HIGH-GRADE PROSTATIC INTRAEPITHELIAL NEOPLASIA (HGPIN) TO PROSTATE CANCER

This study has completed the third and final full year of funded activity. Data collection protocols have been finalized and protocols approved by appropriate IRBs. The majority of work revolved around subject recruitment, data collection, biospecimen collection. Also, significant effort was placed on data management and statistical analyses necessary to achieve specified aims. Accomplishments are described in the following narrative, in parallel with the original Statement of Work.

INTRODUCTION

This work is based on a growing body of laboratory evidence that estrogens contribute to the advancement and detection of prostate cancer. Prostate tumor cells express estrogen receptors, and estrogen exposure may sustain tumor growth as androgen levels decrease with advancing age [1]. It has further been shown that not all estrogens behave alike. Relative to 2-hydroxyestrone (2HE), 16HE appears bind with higher affinity to the estrogen receptor [5], and the relative balance of estrogen metabolites is determined by several enzyme activities. The CYP3A4 and CYP1B1 P-450s are responsible for converting estradiol to the 16HE or 4 HE metabolites, respectively, at the expense of 2HE production. Both CYP1B1 and CYP3A4 are expressed in prostate tissue, a polymorphism of the *CYP3A4* gene in the 5' transcriptional regulatory element (*CYP3A4-V*) has been significantly associated with later age and advanced stage at CaP detection compared to men with the wild-type gene [6]. Similarly, prostate cancer cases may be 3-fold more likely than controls to be homozygous for the *CYP1B1* ^{Val} allele [7]. The goal of this New Investigator Award is to conduct a pilot case-control study investigating the association between molecular markers of estrogen metabolism and diagnosis of prostate cancer (stage II/III) and high-grade prostatic intraepithelial neoplasia (HGPIN), the purported precursor pathologic state to prostate cancer. We will recruit 45 men with HGPIN, 45 prostate cancer cases, and 45 healthy controls free of prostate cancer at biopsy. Genetic polymorphisms in CYP3A4 and CYP1B1 associated with estrogen metabolism will be determined from blood collected from each participant. Data regarding family history of prostate cancer and other risk factors is collected by questionnaire, and habitual dietary intake is measured by in-person interview. We hypothesize that CaP cases will have lower urinary 2HE/16HE and 2HE levels, and higher urinary 16HE, 4HE, E2 and blood E2 levels compared to HGPIN cases or healthy controls. Also, new research finds that prostate cancer is more strongly associated with obesity than previously thought [8-10]. In men, body fat converts androgens to estrogens, and therefore an investigation of body fat and obesity on prostate cancer has direct relevance to the aims of this study.

With completion of the third year of this award, we have met and built upon the 1-Year and 2-Year

objectives specified in our statement of work, including the obtainment of IRB approval, development of questionnaires and other data collection tools. We have expanded our recruitment base to include the Veteran's Hospitals in Nashville and Murfreesboro, TN. Recruitment of study participants has required a great deal of work, but we have exceeded initial recruitment estimates. Data from almost 500 subjects has been entered into a computer database, quality checked, and are ready for analyses. DNA from blood has been extracted, 3 genetic polymorphisms evaluated, and 2 more are in progress. Based on recent research developments described above, we have added analyses of estrogens and prostate cancer in the context of obesity.

BODY

Work accomplished or initiated during each year of this NIA encompasses Statement of Work tasks outlined in Stage 1 (Run-In: Months 1-8)) and Stage 2 (Recruitment and Data Collection: Months 9-26)), Stage 3 (Months 12-30), Stage 4 (24-30 months), and Stage 5 (31-36 months).

Stage 1: Run-In Phase: Months 1-8:

1. Develop all questionnaires, pathology record abstraction forms, and data collection protocols

   Year 1: All forms were developed specifically for this project. Our risk factor questionnaire measures include: demographics; age; sex; race; marital status; education; number of children; medical history and treatment; family health history of prostate cancer; history of other cancers, exercise frequency; and smoking status. (Appendix). Our dietary assessment instrument is the Diet History Questionnaire, recently developed by the NIH and shown to have favorable reliability and validity to other established dietary assessment instruments. We had a computer-assisted program developed to guide the implementation of our dietary assessment instrument. Our Pathology Record Abstraction form records the clinic sites, dates and clinical results of all PSA, other lab assays, and digital rectal exam results, ultrasound results, biopsy history and results, pathological diagnosis (HGPIN, cancer), and zone and distribution of the disease. Similarly, we record any cancer diagnosis, as well as stage and Gleason scale of cancer. We have developed Body Measurement protocols (Anthropometrics), including the methods of measuring and recording measurement of participant weight, height, sitting height, waist, and hips. Our Biospecimen Collection form records blood collection status for purposes of DNA extraction or serology, and urine specimens for estrogen metabolite measurement. All samples are aliquoted and stored frozen as necessary to meet our research objectives. Medication use is recorded on the Medication Use form. Subjects are instructed to bring all current medications and supplements to the data collection meeting, and name of the drug, dosage, and use patterns are recorded. The Interview Checklist ensures that all assigned tasks required during the interview are completed, including a review of eligibility, completion of all data collection protocols, and proper consent of participant.

2. Hire a Research Nurse

   Year 1: For this project, Saundra Motley, RN, was hired to serve as Project Manager. Ms. Motley is responsible for the day-to-day administration. She also has been trained to collect

body size measurements using standard and systematic protocols, as well as in urine and blood collection, sample preparation, and storage protocols.

Year 2: Ms. Motley continued to serve as Project Manager. She is responsible for recruitment, data collection, and day-to-day administration of the project.

Year 3: Ms. Motley continued to serve as Project Manger. She is responsible for recruitment, data collection, and day-to-day administration of the project.

3. Pre-test questionnaires and forms

Year 1: All instrument materials have been thoroughly reviewed, and final versions of all assessment instruments have been produced.

4. Refine questionnaire protocols, as necessary

Year 1: The Baseline Questionnaire was edited to provide a better organization to the questions. We have improved our assessment of dietary intake by developing portion size guides (Appendix), and the addition of several foods common to the Tennessee population (asparagus, okra, squash). We are conducting a sub-study to determine if completion of the dietary questionnaire at home and with a wife/partner provides substantially different food intake scores compared to our in-person interview protocol. The laptop computer program we had developed for this project initially had several 'glitches', but is now working without errors or failures.

5. Finalize IRB application and consent form, if necessary

Year 1: IRB approved consent forms have been obtained from Vanderbilt University, the VA (Nashville), and the US AMRMC (Appendix).

Year 2: We have started to expand the recruitment base to the VA in Murfreesboro TN. Also, we are in negotiations with a large private urology practice to start recruitment at this site.

Year 3: We successfully recruited from all clinical sites. This included the clinics at Vanderbilt University, the VA hospitals in Nashville and Murfreesboro, TN, and a large private urology clinic in Nashville.

6. Develop the study data management systems, including the combination of Teleform, Microsoft Access, Epi-Info, and SAS

Year 1: Our data management system continues to develop, as we recognize and incorporate new technologists into our study. We are able to rapidly identify potentially eligible men receiving care at one of the urology cancer centers through newly implemented scheduling and patient monitoring software in the clinic. This will greatly increase our efficiency to recruit urology patients in the future. New data collection protocols have been developed to fully utilize all resources. Dietary assessment data are entered directly into an ACCESS database using a computer-based dietary assessment interview program created specifically for this project. The ACCESS database may be read by SAS, and merged with other databases for

the purposes of analysis. Additional data-entry programs for questionnaires and forms will follow this model, using ACCESS with subsequent transfer into SAS.

Year 2: Data entry programs for all data collection forms have been developed. Data are entered on a periodic basis throughout the year. About 50% of collected data have been key-punched at this time.

Year 3: Data for 489 subjects have been key-punched and quality checked. This includes double-keypunch entry and evaluation of mean and outlier values. Suspicious values have been checked for accuracy.

7. Become trained in all clinic-site office and administrative procedures

   Year 1: The PI and/or Project Manager have received tours of the urology clinic at Vanderbilt and the VA (Nashville), and understand the operational and administrative procedures of this clinic.

   Year 2: The PI and Project Manager have received tours of the urology clinic at the VA (Murfreesboro), and recruitment at this site has started.

8. Hire and Train Project Director in all clinic based procedures

   Year 1: See #2 above. The position of Project Director and Research Nurse were efficiently combined under Ms. Motley's position in the study.

   Year 2: See #2 above. Ms. Motley continues as Project Coordinator. With expansion of recruitment to the VA at Murfressboro, TN, she is training a VA research nurse in our data collection protocols, in addition to other study responsibilities. Full recruitment from this VA will begin once training is concluded.

   Year 3: See #2 above. Ms. Motley continues as Project Coordinator.

9. Finalize all biological sample collection and storage procedures to be used in the study

   Year 1: All biological sample collection and storage procedures for urine and blood cells and serum are finalized. After obtaining consent, we obtain a urine and blood sample, body size measurements, and participants complete the diet questionnaire. Urine is aliquoted to 9 cryovials, and stored at –80 C. One tube of blood, 5ml (EDTA) is collected for DNA extraction.Two 5ml tubes of blood (No anticoagulants) are collected, centrifuged, and the supernate aliquoted for steroid hormone measurement.

   Year 2: Biospecimen collection and storage protocols are being implemented according to protocols.

   Year 3: Biospecimen collection and storage protocols are being implemented according to protocols.

10. Establish reliability of all laboratory procedures to be used

    Year 1: The genotyping assays for CYP3A4 and CYP1B1 are routine genotyping assays in our group with established reliability. The estrogen metabolite assays have published

reliability and validity. Laboratory reliability will be determined at the time the assays are scheduled to run, during Year 2 or Year 3 of the project.

Year 2: A subset of urine samples have been sent to Dr. Fritz Parl for trial analysis of urinary estrogen metabolite levels. Results from this lab trial were not available at the time of this report.

Year 3: Genotyping for CYP1B1 polymorphism is complete. Genotyping for CYP3A4 polymorphism is currently in progress. This genotyping was delayed somewhat due to a misunderstanding in assay protocol between the PI and service lab, but is now in progress and should be complete within 3 weeks.

11. Establish screening procedures for men with HGPIN, prostate cancer patients, or controls.

    Year 1: Candidates are identified by collaborators in the urology clinics as potentially eligible for our research study. As part of standard recruitment procedures, we mail an introductory letter and brochure to candidates (Appendix). This is followed by a telephone calls (Appendix for script) intended to answer any questions and evaluate interest in the study. If interested and determined potentially eligible, a meeting is scheduled at the study center. The Project Manager confirms eligibility and obtains informed consent (Appendix for consent form) prior to data collection.

    Year 2: Recruitment and eligibility screening procedures were implemented.

    Year 3: Recruitment and eligibility screening procedures are completed.

12. Order supplies necessary for biological sample collection

    Year 1: All supplies necessary for biological sample collection have been ordered and received.

    Year 2: Additional supplies ordered as needed.

    Year 3: Additional supplies ordered as needed.

13. Create complete manual of operations

    Year 1: We have created a manual of operations to organize and record every procedures and protocol in this study. All items in the Appendix are included in the Manual, as well as a copy of the original grant proposal and correspondence with DOD colleagues.

    Year 2: The Manual of Operations continues to be updated as protocols are revised or as administrative documents are received.

    Year 3: The Manual of Operations was updated as needed, and now contains a complete record of all protocols, study documents, and procedures.

<u>Stage 2a: Recruitment of 45 men with high grade intraepithelial neoplasia, 45 men with Stage II/III prostate cancer, and 45 men without CaP or HGPIN at biopsy: Months 8-26</u>

1. Identify 45 men with HGPIN eligible for the study from the urology clinics.

2. Identify 45 men with prostate cancer.

Year 1 and **Year 2**: Study recruitment started January 22, 2003. At the end of Year 1, we had recruited, consented, and collected complete data from 15 participants: HGPIN (n=3), Prostate Cancer (n=7), Controls (n=5). At the end of Year 2, we have recruited and obtained complete data from 129 subjects: HGPIN (n=12), Prostate Cancer (n=55), Controls (n=62).

Year 3: At this time (Year 3), we have data from 491 patients: HGPIN (n=66), Prostate Cancer (n=180), Controls (n=232), and Suspicious (n=13). Approximately 20 more subjects are in various stages of recruitment and data collection. Thus, we have met our recruitment goal of 45 HGPIN patients, and we will be able to conduct analyses comparing prostate cancer, HGPIN, and Controls on parameters of estrogen exposure. This recruitment success is important, as this now is one of the larger clinical and biospecimen databases of HGPIN patients.

Our recruitment rate of eligibles exceeds 93%, a very high success rate in this day and important as we translate our NIA study findings to a full-scale prostate cancer study. We note that recruitment was initially delayed by approximately 4 months due to IRB requirements, thus extension of recruitment efforts into Year 3 was expected.

3. Abstract medical records for health history and pathology data, as described in Methods section of this proposal.

   Year 1 and **Year 2**: We have accessed medical records and abstracted relevant prostate cancer pathology information using our pathology data form.

   Year 3: We have completed an extensive medical chart review of all subjects. Medical charts were reviewed, data recorded on data abstraction forms, and double-keypunched into a computer database. These data include all available pathology information from biopsy. Pathology from prostatectomy patients is included when appropriate.

4. Gain informed consent

   Years 1-3: All participants are consented in an IRB approved manner

5. Among those who say they are willing to participate, determine eligibility using the criteria described in the Methods Section of this proposal

   Years 1-3: Eligibility is determined via telephone interview, and confirmed during the in-person interview.

6. Enroll consecutive eligible men with confirmed HGPIN or prostate cancer.

   Year 1-3: Approximately 93% of eligible HGPIN and prostate cancer patients approached for enrollment have enrolled in our study.

7. Ensure that the spot urine samples and blood samples are collected, processed, and stored in a –80° C freezer at the Vanderbilt University

   Years 1-3: All urine and blood samples are collected and processed using described protocols, then stored in a –80 C freezer at Vanderbilt University.

8. Collect data on lifestyle, demographics, and health (family and personal history), as outlined in proposal

   Years 1-3: Each participant completes a Background Questionnaire, collecting lifestyle, demographic, and medical data. This questionnaire is mailed to participants prior to the clinical visit, and then reviewed by the Project Manager at that time for completeness. Any blank questions or illogical responses are clarified at that time.

9. Pathology record abstractions will be performed

   Years 1-3: The Project Manager abstracts relevant medical and pathology information from the pathology report, and records this information on the Pathology Report Form. This data are periodically key-punched into an ACCESS database.

10. Collect anthropometrics

    Years 1-3: We measure weight, height, waist, hips, and sitting height for each participant using standardized protocols as described. All body measurements are recorded on our Body Measurement Form, and are periodically key-punched into an ACCESS data base.

Stage 3: Data Entry, Verification and Interim Analysis, Months 12-30

1. Assure that all data are immediately read into analytic databases

   Year 2: Data from the diet and physical activity interviews are immediately available for analyses. Data reported by questionnaire, or recorded on the appropriate forms, have been periodically key-punched into ACCESS databases, as work loads permit.

   Year 3: Data entry for analytic study database has been completed.

2. Flag all outlier and illogical responses

   Year 2: All questionnaires are reviewed prior to data entry to identify possible illogical responses. At the time of this report, study recruitment and data collection are in progess. Once recruitment and data collection are closed, data verification and quality control procedures will be implemented.

   Year 3: Outliers and illogical responses were flagged. Questionnaires were checked to confirm or correct values.

3. Verify all outlier and illogical responses, re-contacting participants, if necessary.

   Year 3: Patients were contacted as necessary to resolve any descrepancy.

4. Conduct simple descriptive analyses (e.g., cross-tabulations and univariate statistics)

   Year 3: Simple descriptive analyses completed.

Stage 4: Laboratory Analyses, Months 24 – 30

1. Transfer urine and blood samples to the lab of Dr. Parl for estradiol and estrogen metabolite assays

Year 2: A subset of urine specimens have been transferred to Dr. Parl's lab for preliminary testing of endocrine assays. This will help us evaluate assay quality and variability when urine specimens, such that this analysis may be more rapidly completed for all subjects in Year 3 of the project.

Year 3: Urine samples are available to Dr. Parl's lab. Samples have not been analyzed at this time due to personnel changes in the lab. However, it remains our goal to analyze these samples in accordance with the initial application. Also, we note that the research in the area of urinary estrogen metabolite measurement has progressed, and a post-doc in Dr. Parl's lab is update all analytic procedures to meet these improvements.

2. Transfer blood sample to lab of Dr. Parl for estradiol assay

Year 3: see above

3. Transfer blood samples to lab of Dr. Cai for CYP1B1 and CYP3A4 genotyping assays

Year 3: DNA was extracted from 336 blood samples, including 60 HGPIN patients, prostate cancer patients, and controls. A single 396 well plate was prepared by the Vanderbilt Genetics Core Laboratory, using 0.5 ug DNA were well from 378 subjects. Analysis included 147 cancer patients, 52 HGPIN patients, and 179 controls, frequency matched by age (5 year categories) and race. Genotyping for CYP1B1 L/V was performed by TaqMan assay. Genotyping for CYP3A4 will use this same plate. CYP3A4 genotyping is in progress, and analytic results are expected in 2-4 weeks.

4. Confirm quality control procedures, and repeat assays if necessary

Stage 5: Final Data Analysis, Months 30-36:

1. Perform exploratory analyses to test for adherence to model assumptions

Year 3: Exploratory analyses in progress, but at this time all modeling assumptions and other assumptions necessary for statistical analyses appear to be met.

2. Perform any necessary data transformations to meet statistical assumptions

Year 3: Variables reflecting weight, BMI, WHR, and other anthropometric parameters are scales as continuous variables and also categorized to evaluate inconsistent relationships.

3. Test study hypothesis

Year 3: The primary analysis comparing the prevalence of CYP1B1 genotypes across disease status has been performed. We observed that genetic polymorphisms were not significantly associated with the risk of cancer (OR=1.24, 95% CI (0.78, 2.07) or HGPIN (OR=1.45, 95% CI (0.74, 2.82).

Analyses of CYP3A4 analyses will be completed in a few weeks, once the genotyping is complete. Similarly, analyses of estrogen metabolite levels will be completed once newly hired staff are training in protocols.

4. Conduct post-hoc analyses of study data

Year 3: We have conducted several post hoc analyses looking at the relationship between obesity and prostate cancer. Also, we have extended our genetic analyses to include genetic polymorphisms associated with obesity and prostate carcinogenesis (PPAR-gamma, PPAR-alpha). Consistent prior observations, but a new finding, we observed a marginally significant association between the WHR and HGPIN status. Men in the highest quartile of the WHR were at significantly greater risk of being diagnosed with HGPIN (OR=2.50 1.00, 6.40). To pursue this observation, we have genotyped the peroxisome proliferator activated receptor-gamma and alpha, for alleles previously associated with obesity. Associations between PPAR genetic polymorphisms and prostate cancer or HGPIN risk were not statistically significant, however we are pursuing sub-analyses to determine if there is modification by weight or other body size parameters.

5. Prepare manuscripts
Year 3: Manuscript preparation has not yet started, but we anticipate several manuscripts from this work.

6. Archive datasets for future analyses and future patient follow-up
Year 3: All datasets are backed-up daily on the server for security and storage

7. Plan for future studies
Year 3: This work has motivated several ongoing, submitted, and proposed grant proposals.

There has been no work accomplished within stage 5 of the Statement of Work.

KEY RESEARCH ACCOMPLISHMENTS in Year 3

- IRB approvals maintained
- Manual of Operations maintained
- Participant recruitment, including consent, recruitment, eligibility, data collection, biospecimen collection, and storage protocols.
- Exceed original recruitment and data collection projections.
- Abstraction of pathology data from medical records

- Data-entry of all data and evaluate data quality
- Completed genotyping of CYP1B1, and CYP3A4 genotyping in progress
- Completed genotyping of PPAR-gamma, PPAR-alpha, and CYP19 supplemental to the original protocol
- Urinalysis of endocrine study delayed due to a change in personnel within service lab. However, we anticipate analyses within 3-6 months.
- The key research accomplishment is that this study has initiated a prostate cancer research program across Vanderbilt, Meharry Medical School (A HBCU) and across the greater Nashville commuity that will likely pay dividends for many years.

REPORTABLE OUTCOMES

We continue to analyze these data and test our hypotheses regarding the role of genetic markers of estrogen exposure and prostate cancer risk. Toward this end, a student with a research interest in prostate cancer racial disparities is now using these data for her master's research project, and she has plans to continue in this vein and to seek her PhD.

This research has been used to demonstrate recruitment feasibility of prostate cancer patients for four grant applications. The first was submitted as a pilot project in a Prostate Cancer SPORE application submitted to the NIH. The objective was a case-control study extending this recruitment protocol to investigate additional biomarkers of prostate cancer risk (SPORE was not funded). In the second application, Dr. Fowke submitted a pilot intervention to the American Institute of Cancer Research to investigate the effects of diet on PSA levels among prostate cancer patients (funded and project is initiated). In the third project, Dr. Fowke will submit an IDEA award to the DOD to investigate the effects of indole-3-carbinol or Crucifer vegetable intake among men with PSA recurrence following prostatectomy (unfunded). The fourth was an exploratory project to the DOD investigating serologic markers of obesity.

Dr. Fowke was recently listed among collaborators in a DOD funded Prostate Cancer Research Center grant, awarded to Meharry Medical School (PI: F. Ukoli). Dr. Ukoli and Dr. Fowke are collaborating on Dr. Ukoli's initiatives to conduct a diet intervention among African-American men, and Dr. Ukoli will conduct analyses using data collected under this protocol in the future to provide preliminary data for future grant applications.

CONCLUSIONS

In Year 3, recruitment and data collection protocols were fully completed. We have data and biospecimen collection from 498 subjects, with data collection from 20 more in progress. We recruit 93% of eligible subjects to this study, a very respectably high recruitment rate. We note that certain laboratory assays are not completed in time for this report. This NIA has provided the opportunity for Dr. Fowke to submit several prostate cancer research grants. Statistical analyses will continue to investigate hypotheses linking steroid hormone metabolism to prostate cancer progression. CYP3A4 genotying is in progress, and the urinalysis of estrogen levels will be performed once Dr. Parl's laboratory has re-trained new personnel to complete the assay. We are prepared to submit this report as is, or to request a no-cost extension to complete the stated work items.

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